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14. ABSTRACT

We are testing, in prospective studies from Hopkins (Brady) and Harvard (PHS, HPFS), whether the combination of telomere length variability in prostate cancer cells and short telomere length in cancer-associated stromal cells is an independent prognostic indicator of poor prostate cancer outcome. We are automating the method for measuring telomere length and will seek optimal prognostic cutpoints. In Year 1, Hopkins purchased a fluorescence slide scanner and image analysis software using donor funds. In Year 2, we submitted a revised SOW and budget for the use of this scanner/software for telomere length determination for this project. Approval was received in 7/2014. We hired and trained a dedicated technician to support the automation/optimization. We developed optimized protocols for fully automated slide scanning and multi-channel acquisition of fluorescent images. Further, we used the image analysis software to automate the segmentation of individual cell nuclei and telomere FISH signals. We showed that telomeres from each individual cell can be extracted using a dot detection algorithm. We have confirmed that a tissue microarray format may be utilized with this approach.

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INTRODUCTION: Currently used clinico-pathologic prognostic factors are imperfect predictors of outcome in the men with clinically localized prostate cancer, the majority of men diagnosed today. Thus, tissue-based biomarkers that significantly enhance the predictive power are urgently needed to improve treatment and surveillance decision-making for these men. To address this pressing clinical need, we have assembled a multidisciplinary prostate cancer research team from Johns Hopkins and Harvard to validate and optimize a novel tissue biomarker of prognosis for men with clinically localized prostate cancer that we recently identified – telomere length variability in prostate cancer cells combined with short telomere length in cancer-associated stromal cells. In our prior work, men with this combination had a substantially higher risk of dying of their prostate cancer compared with men without this combination. Equally importantly, men without this combination rarely died of their prostate cancer over 10 years. Key next steps to realize the great translational potential of telomere length as an independent prognostic tissue biomarker are optimized biomarker assessment and validation. Thus, our aims are to: 1) Optimize the method for assessing telomere length by FISH using a high-throughput approach to yield a test feasible for the clinical setting. 2) Validate our compelling findings in two other cohort studies on prostate cancer outcomes: a) men surgically treated and followed for lethal prostate cancer; and b) men surgically treated and followed for prostate cancer recurrence. 3) Determine optimal biomarker cutpoints for prognosis.

BODY: This work is being performed collaboratively by two institutions: Johns Hopkins Bloomberg School of Public Health and Harvard School of Public Health. In Year 1, we obtained all required IRB approvals for both the Brady prostate cancer recurrence Study (Hopkins), the Physician's Health Study (PHS) and the Health Professionals Follow-up Study (HPFS, Harvard), including from the DOD IRB (**Task 1 completed**). Drs. Platz and De Marzo previously created the Brady prostate cancer recurrence nested case-control study (Brady study; in part with prior DOD funding to Dr. Platz at Hopkins) and associated tissue microarrays (TMAs). This TMA set is now part of the Prostate Cancer Biorepository Network (PCBN). For equitable use and tracking purposes, during Year 1, we applied for access to these TMAs and received approval from the PCBN. For the Brady study, we pulled the recurrence TMAs (N=16 TMAs, which includes 524 cases and 524 controls) and cut and mounted the sections (**Task 4b completed**) for staining for telomere-specific FISH, cytokeratin 903 immunofluorescence, and for DAPI.

The PHS and HPFS are existing cohort studies, and TMAs have been constructed for those participants who underwent a radical prostatectomy with other funds, including previous DOD funding (to Dr. Mucci at Harvard). In Year 2, we received sections from the 6 PHS TMAs and 3 HPFS TMAs that had not yet been constructed when we conducted our prior work on the telomere biomarker and that served as the preliminary data for the proposal for the current study (**Task 4a completed**).

In Year 1, the Johns Hopkins investigators purchased a new state-of-the art fluorescence slide scanner and associated image analysis software from Tissue Gnostics using donor funds (**Task 2a completed**). This new system uses the TissueFAXS Plus (Tissue Gnostics, Vienna, Austria) microscopy workstation for slide-

based cytometry of tissue sections and TMAs. The microscope is a Zeiss Z2 Axioimager with high quality optics applicable for fluorescence imaging. The microscope is fitted with the following filter sets: DAPI, Alexa 488/Cy2, Alexa 568/Cy3, and Alexa 633/Cy5. The system contains an ultra-precise motorized stage for 8 slides for high throughput scanning. In addition, a separate image analysis workstation contains a high-performance computer workstation (HP Z420 configured with 6 cores) and includes the TissueFAXS 4.0, TissueQuest 4.0, and HistoQuest 4.0 software modules (Tissue Gnostics).

On May 6, 2014 (during Year 2), we submitted a revised SOW and budget to reflect the use of the new fluorescence slide scanner and associated image analysis software at Johns Hopkins, which were recommended for approval on May 29, 2014. The fully executed basic award with these modifications became effective on July 22, 2014.

In Year 2, we have developed optimized protocols for fully automated slide scanning and multi-channel acquisition of fluorescent images using a 40X oil objective. As shown in Figure 1, we are able to image prostate tissues that have been fluorescently stained with a Cy3-labelled telomere specific peptide nucleic acid probe (red), immunolabeled with a prostate basal cell-specific anti-cytokeratin primary antibody and detected with a fluorescent secondary antibody conjugated to Alexa Fluor 488 (green), and counterstained with DAPI to detect the nuclei (blue).

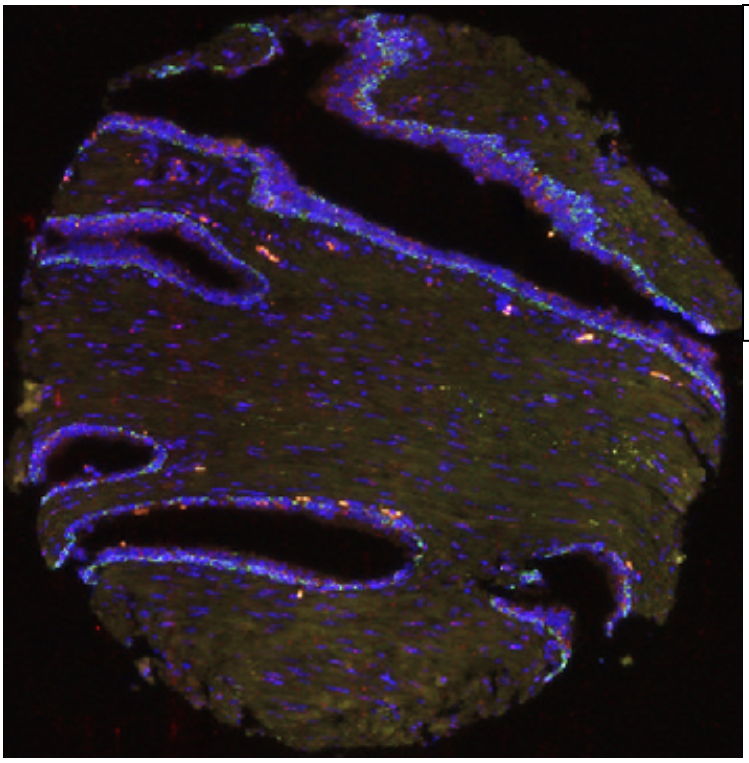

Figure 1. Example of a single prostate TMA spot containing normal prostate glands embedded in prostatic stroma. DNA is stained with DAPI (blue), telomeric DNA is stained with a Cy3-labeled telomere-specific PNA probe (red), and basal cells are demarcated with a basal cell-specific cytokeratin antibody (green). Original magnification × 400.

In Year 2, we have also utilized the Tissue Gnostics image analysis software to automate the segmentation of individual cell nuclei and telomere FISH signals (**Task 2b completed**). Figure 2 below shows a normal prostate gland with surrounding stromal cells; the nuclei are labeled in blue, telomeres in red, and the normal basal cells in green. Individual cells within the orange circled region of interest are automatically identified and outlined (here, in green) through a nuclear segmentation algorithm by the analysis software. Each specific cell type within the prostate may be analyzed. As an

example, the subset of basal cells are automatically identified, based on their protein expression pattern, and outlined in red by the software. These individual basal cells are then isolated for eventual extraction of their telomere data.

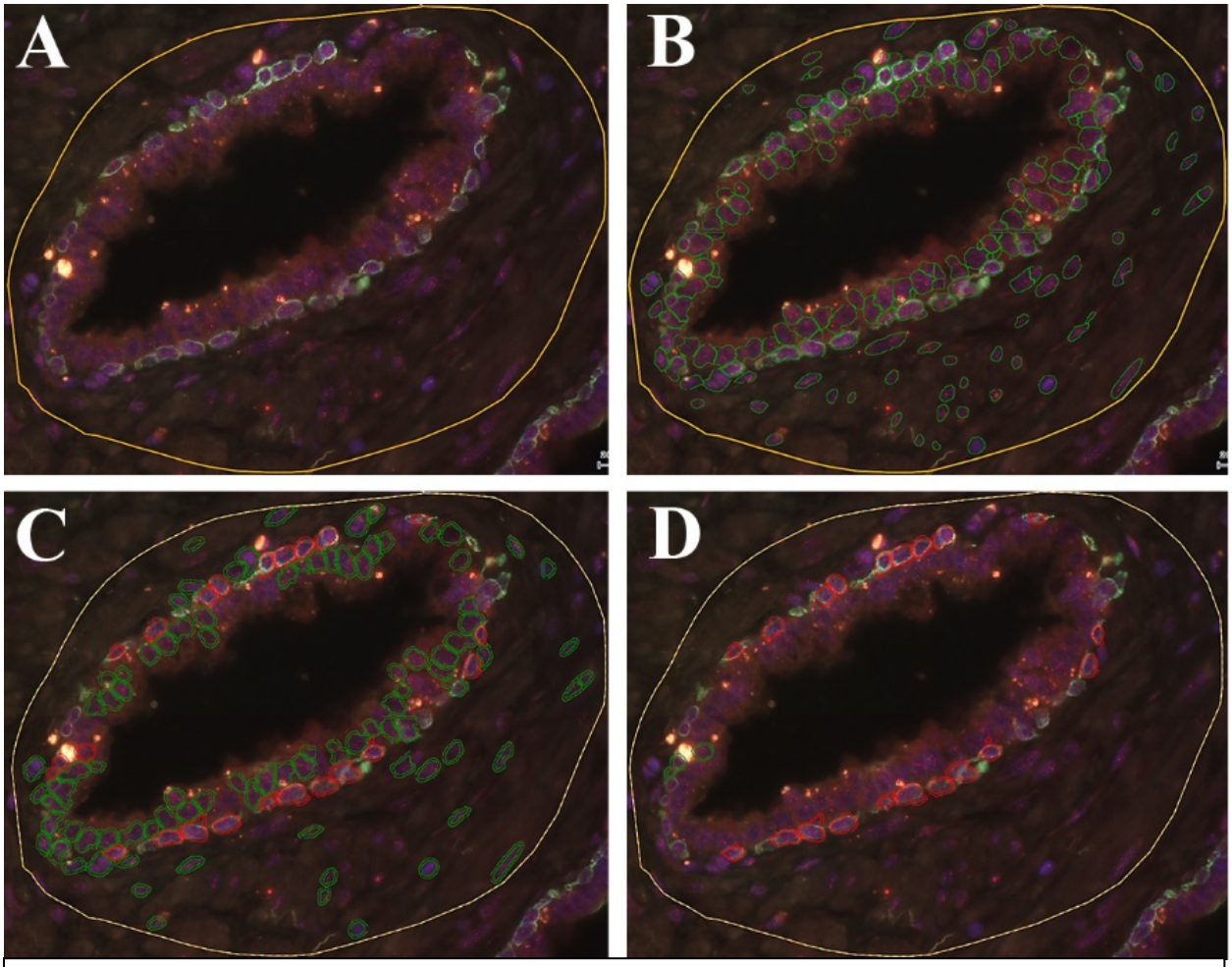

Figure 2. Example of how a specific cell population within a normal prostate gland may be identified.

The telomeres from each individual cell can be extracted using a dot detection algorithm. As shown in Figure 3, the telomere fluorescence channel shows the individual telomere signals as punctate dots within the nucleus. The telomeres within the circled region of interest are automatically identified and outlined (in yellow) by the Tissue Gnostics image analysis software and can be quantified as the ratio of the total intensity of telomeric signals divided by the total DAPI sum area; thus, determining the telomere length on a per cell basis.

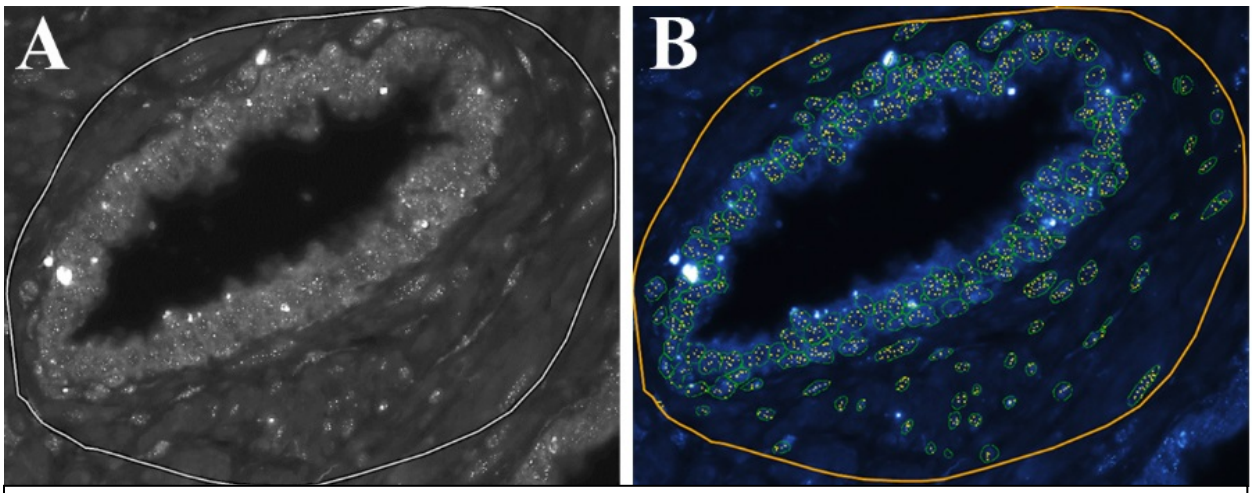

Figure 3. Example of how individual telomeres within each cell are identified and quantitated.

Finally, we have confirmed that a tissue microarray (TMA) format may be utilized with this approach. A portion of a prostate cancer TMA scanned with the Tissue Gnostics scanning fluorescence microscope is shown in Figure 4.

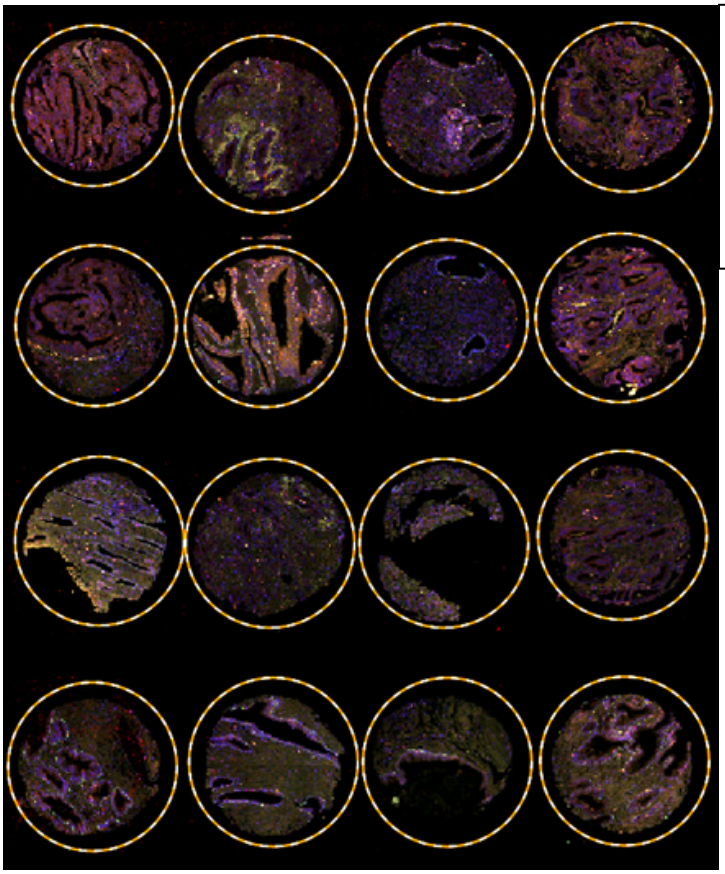

Figure 4. Example area of a prostate TMA. DNA is stained with DAPI (blue), telomeric DNA is stained with a Cy3-labeled telomere-specific PNA probe (red), and basal cells are demarcated with a basal cell-specific cytokeratin antibody (green). Original magnification × 400.

KEY RESEARCH ACCOMPLISHMENTS: None directly from this project to date (see above).

While we are pursuing the aims of the current DOD grant, our telomeres and prostate cancer team continues to conduct research addressing the aims and related questions using the data collected under our two prior DOD grants on telomeres (W81XWH-06-1-0052, W81XWH-05-1-0030).

As previously reported, our team studied telomere length in circulating leukocytes as a possible biomarker for the risk of prostate cancer. The final references is as follows: Hurwitz LM, Heaphy CM, Joshu CE, Isaacs WB, Konishi Y, De Marzo AM, Isaacs SD, Wiley KE, Platz EA, Meeker AK. Telomere length as a risk factor for hereditary prostate cancer. Prostate. 2014. 74(4):359-364. This study was primarily funded by a prior DOD grant (W81XWH-06-1-0052).

In addition, the team, with Dr. Corinne Joshu (co-I on current DOD award) taking the lead, presented the following abstracts using data from our prior DOD award (W81XWH-05-1-0030):

Joshu C. et al. Pre-diagnostic obesity and inactivity are associated with shorter telomere length in prostate-cancer associated stromal cells (proffered abstract presentation and poster). 12th AACR International Conference on Frontiers in Cancer Prevention Research. October 27-30, 2013, National Harbor, MD. A manuscript is drafted and is under co-author review.

Joshu C. et al. Current or recent smoking is associated with more variable telomere length in prostate-cancer associated stromal cells and prostate cancer cells (poster). 20th Annual Prostate Cancer Foundation Scientific Retreat. October 24-26, 2013, National Harbor, MD.

Joshu C. et al. Leukocyte telomere length is not correlated with telomere length in benign prostate-cancer associated cells or prostate cancer cells (poster). 21st Annual Prostate Cancer Foundation Scientific Retreat October 23-25, 2014, Carlsbad, CA.

REPORTABLE OUTCOMES: None

CONCLUSIONS: None to date, as consistent with the Statement of Work.

REFERENCES: None

APPENDICES: None

SUPPORTING DATA: None